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13. ABSTRACT *(Maximum 200 Words)*
It is known that the actions of estrogen in mammary development are mediated primarily by the estrogen receptor alpha, but it is not known which estrogen receptors, those in stroma or those in epithelium mediate mammary development. Our purpose is to probe this question by constructing transgenic mice in which wild type human estrogen receptor (hER) and mutants of the receptor that are super-active either at the classical ERE or alternative AP-1 pathway are selectively expressed in epithelium or stroma. A further purpose is to explore the importance of the AP-1 versus the classical pathway in estrogen effects on ductal development. In the first year of this project we have constructed vectors for expression of wild type and mutant human estrogen receptors in the mammary epithelium of transgenic mice, and have made a pilot study of the effects of expressing these receptors in the reproductive epithelium of transgenic mice. The mice transgenic for a human ER super-active at AP-1 show gross abnormalities and hyperproliferation of the reproductive tract.

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Introduction:

The subject of this research project is the action of estrogen in mammary ductal development. It is known that the actions of estrogen in mammary development are mediated primarily by the estrogen receptor alpha, but it is not known which estrogen receptors, those in stroma or those in epithelium mediate mammary development. Our purpose is to probe this question by constructing transgenic mice in which wild type human estrogen receptor (hER) and mutants of the receptor that are super-active either at the classical ERE or alternative AP-1 pathway are selectively expressed in epithelium or stroma. A further purpose is to explore the importance of the AP-1 versus the classical pathway in estrogen effects on ductal development.

Body:

We divided up this application into two tasks:

Task 1. To Analyze whether estrogen treatment causes mammary ductal hyperplasia in transgenic mice in which human estrogen receptor and pathway specific super active varients thereof are over-expressed in mammary stromal fibroblasts, adipocytes and epithelial cells (months 1-36).

-Construct vectors that will allow expression of human ER and super-active varients of ER in the mammary stromal fibroblasts and other mammary tissues of transgenic mice (months 1-12).

-Create mice carrying the expression vectors for human ER and test them for expression of human ER in mammary gland and other tissues (months 6-24).

-Determine whether mice that express high levels of either wild type or super-active hERs in mammary compartments develop ductal hyperplasia (months 18-36).

Task 2. To determine whether ERKO transgenics that have hER expression in specific tissues of the mammary gland display restored mammary ductal development in response to estrogen

-Establish a colony of ERKO +/- mice (months 1-12)

-Bred these mice to transgenics expressing human ER (months 12-24)

-Analyze mammary development in ERKO -/- transgene offspring (months 18-36)

For the first year we have made progress on three fronts, vector construction, pilot studies in mice, and establishment of the ERKO colony,

Vectors. In our initial constructions we have used the wild type human estrogen receptor, (hER) and a super active mutant, hER K206A, which increases the efficiency of ER action at AP-1 target genes, but not not increase the efficiency of ER at classical EREs. The behavior of this mutant in transfected cells in culture is shown in the appended manuscript by Anderegg et al.. At AP-1 containing target genes, such as the collagenase gene, and the Cylin D1 gene, the K206A hER is more than 10 fold more efficient than wild type in inducing gene expression. Yet, if anything, the K206A mutant is weaker than wild type in inducing gene expression at target genes with an ERE.

We have made two sets of vectors that should direct expression of wild type and super-active human estrogen receptors to the epithelial cells of the mammary gland of transgenic mice. Each of these uses the Mouse Mammary Tumor Virus (MMTV) promoter to drive transcription. One set uses a standard MMTV transgene vector developed by Dr. William Muller and his colleagues and that has been used for making numerous transgenic mouse lines that express various proteins in mammary epithelium (Cardiff and Muller, 1993, (Dankort and Muller, 1996, Guy, et al., 1994, Li, et al., 1997). The map of the vector is show in figure 1 overleaf. Notice that the vector uses the SV40 small t antigen splice and poly adenylation signal that has been claimed to limit expression somewhat in transgenic mice.

Although these vectors do drive expression of human ER properly in transfected mammary cells as shown in the figure below, their level of expression is low. To ensure high level expression, we have therefore undertaken the development of an improved vector system, which is again outlined in the figure. The improved vector has the beta globin small intron proceeding the hER

coding sequence which is followed by the human growth hormone poly A addition signal and is shown in Figure 2 overleaf. A classical study by Brinster and colleagues suggests that the small intron and growth hormone poly A greatly aid expression in transgenic mice (Brinster, et al., 1988). In addition, we have flanked the expression cassette on both sides with an insulator sequence derived from the chicken beta globin gene. Although this sequence has not been tested in transgenic mice, it does confer much more reliable expression in stable transfection into tissue culture cells (Bell, et al., 1999).

The vectors driving wild type and mutant hERs are currently being injected into mice to create the transgenes.

Pilot Study. We have made a pilot study of our strategy to overexpress human ERs in mice in collaboration with Jeffrey Arbeit's lab here at UCSF. Dr. Arbeit heads the transgenic facility at the UCSF Cancer Research Center and his own lab has had extensive experience with the use of the Keratin 14 gene promoter in transgenic mice (Arbeit, et al., 1996). The K14 promoter drives expression in selected epithelial tissues including the skin and the lining of the lower reproductive tract in females. We therefore made expression vectors of hER and K206A driven by the K14 promoter and introduced these vectors into transgenic mice. The results of this experiment are reported in detail in the appended manuscript.

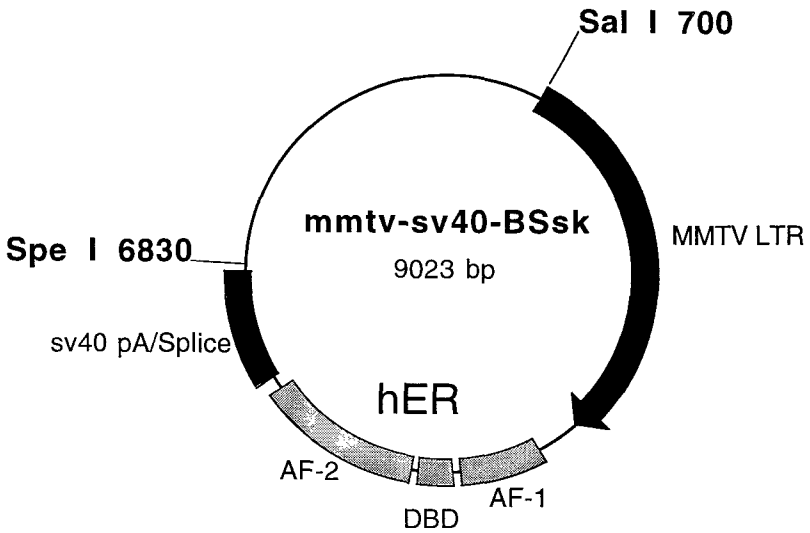
In brief, human wild type ER was very well expressed in the cervical and vaginal epithelium of the single line of mice with this construct. No aberrations could be seen either in gross morphology of the reproductive organs, or in histological examination. There was a small and variable increase in proliferation, evidenced in staining with antibody to the Proliferating Cell Nuclear Antigen (PCNA), and an increase in Cylin D1 expression in the epithelium. Two lines of mice carried the K14:K206A transgene gave lower levels of expression of the hER. However, in contrast to the mice expressing wild type hER, the K206A mice showed gross aberrations of the reproductive tract. In both lines the cervix and vagina of aged animals is

greatly enlarged (5-10 times normal). This appears due to hyperproliferation of the epithelial cells as indicated by PCNA staining,. There is a dramatic increase in Cyclin D1 expression thoughout the epithelium.

Since the wild type ER was without these effects, yet was expressed at a higher level than K206A, these results suggest that a feature of the K206A mutant must be responsible for the effects on the reproductive tract. We believe that these are due to the ability of this receptor to activate target genes with AP-1 sites, These results are thus in line with expectations that the ER-AP-1 pathway is important for proliferative effects of estrogen.

ER alpha knock out mice (alpha ERKO) . We have received a breeding pair ERKO heterozygous mice from Dennis Lubahn and are building up the colony (Lubahn, et al., 1993).

Figure 1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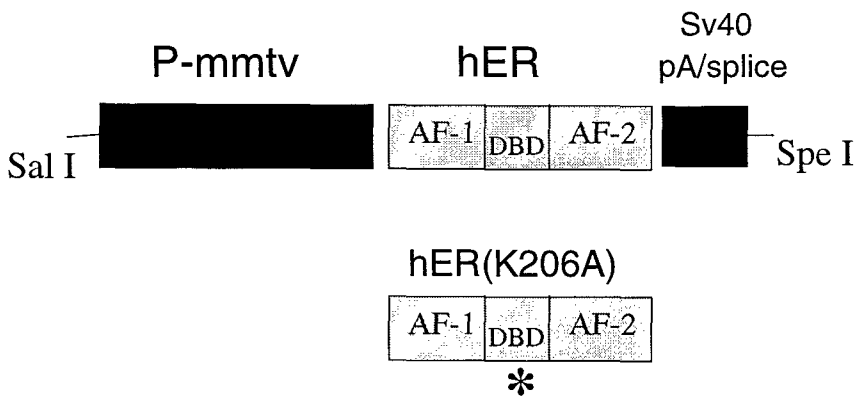
hER
AF-2
DBD
AF-1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AF-2
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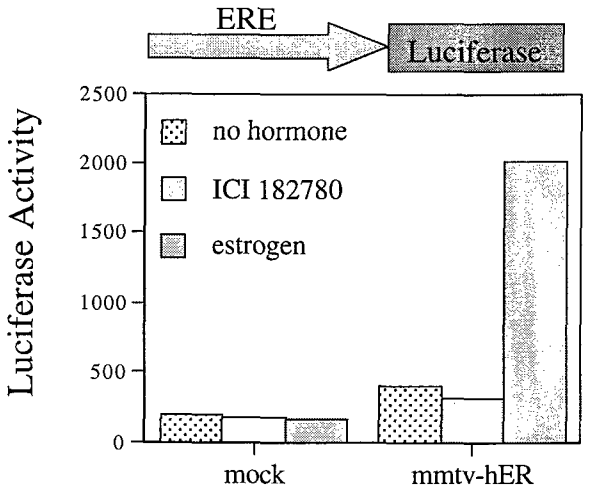
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estrogen
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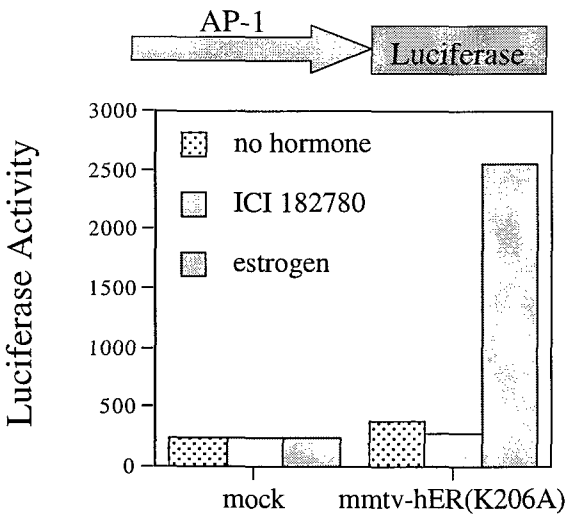
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Luciferase Activity
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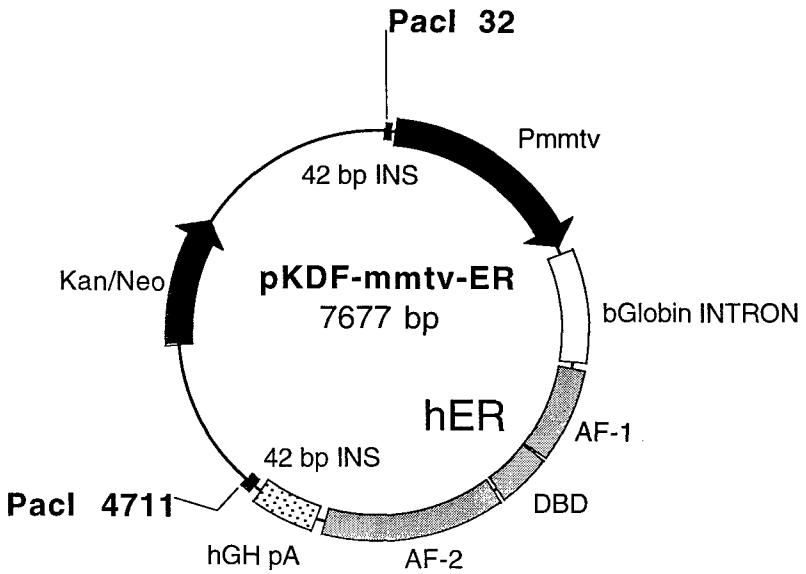
1000
500
0
no hormone
ICI 182780
estrogen
mock
mmtv-hER(K206A)

Figure 2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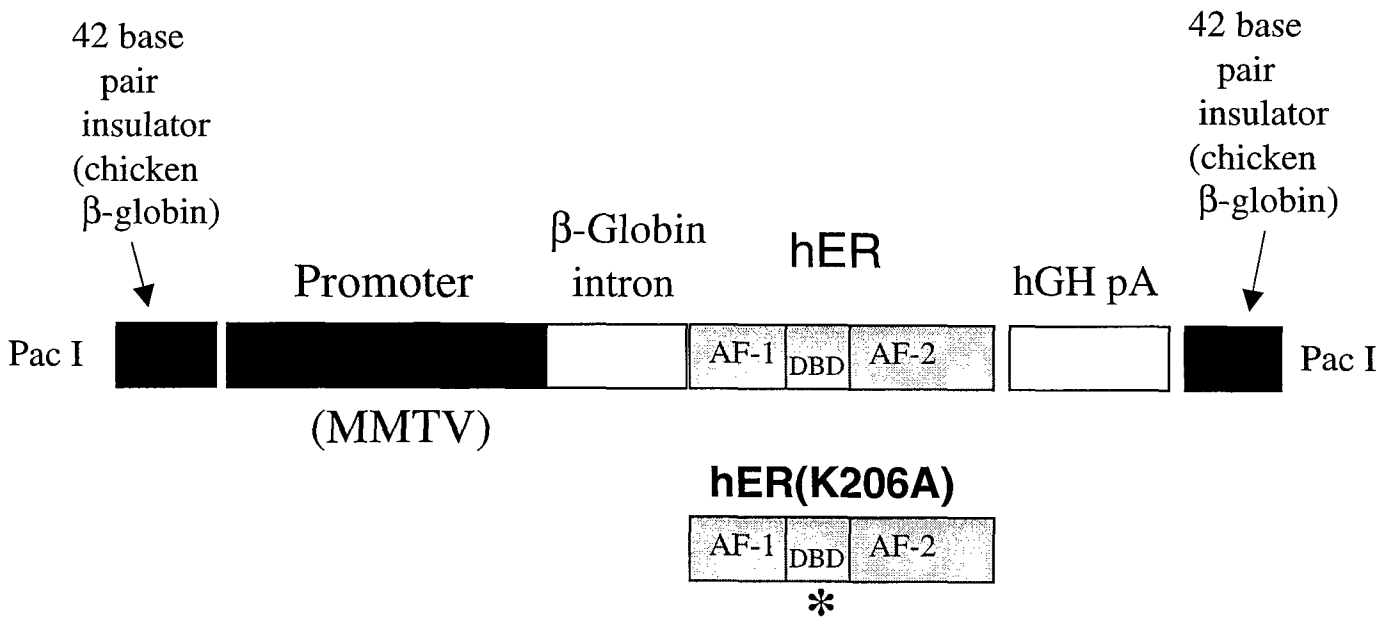
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β-Globin intron
hER
42 base pair insulator (chicken β-globin)
Promoter
hGH pA
Pac I
AF-1
DBD
AF-2
Pac I
(MMTV)
hER(K206A)
AF-1
DBD
AF-2
*

Key Research Accomplishments:

- Demonstrated that a point mutation in the human estrogen receptor allows the receptor to be super-active in just the pathway of action that leads to target genes with AP-1 elements.

- Demonstrated that expression in the reproductive tract of mice of the human estrogen receptor super-active at AP-1 targets leads to hypertrophy and hyperplasia.

Reportable Outcomes:

Manuscripts

One manuscript to be submitted, "The estrogen receptor alpha mutant, K206A, superstimuates AP-1 trans-activation and produces cervico-vaginal hypertrophy and hyperplasia in transgenic mice." This is attached.

Abstracts-

Nuclear Receptors 2000, "A point mutation in the ER DBD separates the AF-dependent from the AF-independent pathways of AP-1 stimulation and generates an abnormal phenotype in transgenic mice."

R. Uht, B. Anderegg, P. Webb, C. Anderson, J. Arbeit, and P. Kushner.

Talks.

Nobel Symposium- Karlskoga Sweden June 1999

Gordon Research Conference on Breast and Prostate Cancer August 1999

Merck Symposium on Estrogen Action, Philadelphia November 1999

University of Colorado Health Sciences Cancer Research Mini Symposium, March 2000

University of California San Francisco, Breast Oncology Group Seminars, June 2000

Patents;

One patent has been applied for."Expression of human steroid receptors in transgenic animals. UC case no. 99-382 Jeffrey M. Arbeit et al. Inventors.

US Patent Application No. 09/365,614

Conclusion:

The conclusions from this first year of research are of course tentative. We can say that point mutations in a single amino acid in the DNA binding domain of the human estrogen receptor confer super-activity selectively on AP-1 targets, without conferring super-activity at target genes with classical EREs. This pattern is seen in a variety of cells in tissue culture.

When expressed in the epithelial cells of the reproductive tract of transgenic mice the mutant human ER confers hypertrophy and hyperplasia of the cervix and vaginal epithelium. Of course, we are eager to see what will happen when the same receptors are expressed in the mammary gland of transgenic mice. Then, we will be able to draw some important conclusions with regard to estrogen action in breast cancer. However, from the results at hand it appears that stimulation of the ER-AP-1 pathway leads to turn on of Cyclin D1, hyperproliferation, hyperplasia, and hypertrophy of the lower reproductive tract. Importantly, the wild type ER does not have these effects, suggesting that the AP-1 ER pathway is alone responsible.

"So What?" If expressing the K206A mutant human ER in the mammary gland also leads to hyperproliferation, it will suggest that in hormone dependent breast cancer and premalignant states, the pathway of hormone action leads to AP-1 regulated target genes. "So What?" Well once we know the pathway, we can take steps to block hormone action, and maybe some day develop better antiestrogens to treat or prevent breast cancer. Do we still want to ask, "So what?"?

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Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S. and Smithies, O. (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A, 90,11162-6

The estrogen receptor α mutant, K206A, superstimulates AP-1 *trans*-activation and produces cervico-vaginal hypertrophy and hyperplasia in transgenic mice

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Estrogen receptor ERα activates gene expression by binding estrogen response elements in DNA or by potentiating transcription factors such as AP-1. The latter pathway has been implicated in estrogen stimulation of cell proliferation. We show here that a point mutation in the human ERα, K206A, while leaving transcriptional activation through estrogen response elements relatively unchanged, dramatically increases its ability to stimulate transcription through

AP-1. To determine the effect of selectively amplifying the ER pathway through AP-1 , we generated transgenic mice expressing human ERα.K206A via the keratin 14 promoter, which confers expression in epithelial cells including those of the lower reproductive tract. Female mice expressing hERα. K206A have vaginal and cervical hypertrophy with vaginal squamous hyperplasia by 12 months of age. Non-transgenic controls and mice expressing the wild type receptor via the keratin 14 promoter failed to develop such reproductive tract abnormalities. These results suggest that overstimulation of alternative target genes by ER leads to dysregulated growth in an estrogen target tissue.

Estrogen receptors (ERs) activate expression of classical target genes that contain estrogen resonse elements (EREs) and also alternative targets genes that contain AP-1 sites [1-3], SP-1 sites ([4], and refs therein), and others (see, e.g. [5]) . The ER binds directly to EREs through its DNA binding domain (DBD) and activates transcription through the recruitment of coactivators via its activation functions, the constitutive AF-1 in the amino terminal domain, and the hormone activated.AF-2 in the C-terminal ligand binding domain (LBD). ER activates at AP-1 by protein-protein interactions and is independent of DNA binding. ER action at AP-1 is usually less dramatic than at consensus EREs, but my nonetheless be important as the target genes implicated in estrogen stimulation of cell proliferation, such as the Cyclin D1 gene, have AP-1 or related sites and not EREs [6,7]. ER-estrogen stimulation through AP-1 is also mediated by the ER AF-1 and AF-2 [2]. The ability to stimulate transcription at AP-1 sites is unique to the ER among nuclear receptors, most of which repress transcription at AP-1 sites in the presence of hormone. A point mutation at the base of the first zinc finger of the DBD in the glucocorticoid (GR), thyroid (TR) and other receptors, however, has been shown to convert each of them from an inhibitor to a stimulator of AP-1 activity [8]. We therefore sought to determine the effect of the homologous mutation in the ERα, ER.K206A (Fig. 1A).

ERα K206A super-stimulated a target genes with an AP-1 site, such as the human collagenase promoter (Fig. 1b) or the Cylin D1 promoter (not shown). In each case

K206A was almost ten fold more active than wild type ER. ERα.K206A had normal activity at a variety of ERE target genes including the collagenase promoter with the AP-1 site substituted by a consensus ERE (Fig. 1b right panel), and was weaker than normal at SP-1 targets (data not shown). The K206A mutant is super active in a wide variety of cell types (Fig. 1C). We conclude that, the K206A mutation confers super-activity at AP-1 targets, but not at an ERE.

Mutation of the AF-2 function eliminates the ability of K206A to activate at AP-1, in cells in which ER AF-2 predominates (Fig. 1D). The mutation does not interfere with expression of the mutant receptor as the AF-2 mutant of K206A, like similar ER mutants, is a dominant negative inhibitor of ER action at an ERE (data not shown) In cell types in which ER AF-1 is strong, K206A requires the AF-1 function for full activity (data not shown). Thus K206A stimulates AP-1 targets, through its AF functions, similar to the wild type ER.

Activation of AP-1 target genes is a critical event in proliferation, and tumor genesis. For example, transgenic mice expressing a dominant negative mutant of the AP-1 family member c-jun in the epidermis have reduced skin thickness, and are protected from chemically induced epidermal carcinogenesis. Stimulation of AP-1 targets by estrogen- bound ER has been implicated in the stimulation of cell growth by estrogen ([1,2,9,10], and refs therein). Since the squamous epithelium outlining the vagina and lower cervix proliferates in response to estrogen, we investigated the effect of the K206A mutation on this response. We engineered mice in which human ERα (wild type) and ERα.K206A were targeted to the cervico-vaginal squamous epithelium, by expression through the human keratin 14 (K14) promoter (K14-ERα and K14-ERα.K206A, respectively) (Fig. 2).

Two independent lines of each transgenic model, K14-ERα and K14-ERα.K206A, were established. Female mice were aged for up to 24 months. Thirty of a total of 36 (83%) K14-ERα.K206A females aged for more than 6 months spontaneously developed striking perineal swelling and distention (Fig. 2a, bottom). A similar phenotype was never observed in age-matched K14-ERα transgenic mice or non-transgenic controls (Fig. 2a, top).

Dissection revealed that the K14-ERα.K206A transgenic females developed vagino-cervical hypertrophy and prolapse, that in some instances was pronounced (Fig. 2 b right panels). Histological sectioning revealed epithelial hyperplasia as well as stromal fibrosis (Fig. 2c). No histopathological abnormalities were evident in non-transgenic or K14-ERα transgenic animals.

To rule out that development of the observed hyperplastic changes in K14-ERα.K206A transgenic mice compared to K14-ERα transgenic animals was due to differential transgene expression, immunohistochemistry (IHC) was performed using a human ERα-specific antibody. Transgene-expressing nuclei were much more frequent in the vagino-cervical epithelium of K14-ERα than K14-ERα.K206A transgenic mice (Fig. 2c). Western blotting and immunostaining of cells transfected with either human ERα or human ERα.K206A determined that the antibody used for IHC had equivalent sensitivity to detect either transgene (data not shown). Consequently, the abnormal phenotype of transgenic mice bearing the mutant ERα cannot be explained by higher or more widespread distribution of transgene expressing cells in K14-ERα.K206A than in K14-ERα transgenic mice. We conclude that the striking phenotype of the K206A mice is due to the mutation itself.

K206A mice have an increase in epithelial cell layers and papillations at the epithelial-stromal interface (Fig. 3a) and have higher and more frequent expression of proliferating cell nuclear antigen (PCNA) compared to age-matched K14-ERα transgenic and non-transgenic control mice (Fig. 3b). Therefore, the development of an abnormal vaginal phenotype in K14-ERα.K206A transgenic mice 12 months of age or older is likely due to increased epithelial proliferation.

To determine whether ERα.K206A increased transcription through an AP-1 site in cervico-vaginal epithelium of transgenic mice as previously shown in reporter assays *in vitro*, we determined protein expression of cyclin D. Cyclin D1 expression is known to be regulated by estrogen despite the lack of classical EREs as noted above. Cyclin D protein expression was in good concordance with the PCNA data: In K14-ERα.K206A cervico-vaginal epithelium Cyclin D was more abundant and more widely distributied than in non-transgenic or in wild type ERα transgenic tissues.

Cyclin D was expressed in several suprabasal cell layers (Fig. 3c, top). Thus, ERK206A expression leads to increased cyclin D expression in vivo. This observation accords both with our *in vitro* findings of increased *trans*-activation of AP-1-responsive genes through ERα.K206A expression as well as the recently proposed model of cyclin D1 expression depending on AP-1 factors.

We describe a mutant in the human ERα with over-activation of transcriptional activity at AP-1 sites, but normal activity at EREs. When expresssed in squamous epithelial cells of transgenic mice the mutant ER elicits markedly increased proliferation of the cervico-vaginal epithelium, a known estrogen target. Since the wild type human ER does not confer such a phenotype, we conclude that a feature of the mutant, most likely it's selectivity for AP-1 and related targets is responsible. Thus, in accordance with the predictions made from studies of estrogen action in cultured cells ER action at alternative response elements plays an important role in mediating the proliferative effects of estrogen in vivo.

Gain of AP-1 function has been implicated in cervical carcinogenesis . The data presented here , that ERα signaling at AP-1 sites in cervico-epithelial squamous cells contributes to increased proliferation, may indicate an association of ER action at AP-1 with neoplastic events of the cervix. The transgenic mutant ERα mouse model described here may thus foster development and validation of pharmacological agents interrupting ERα/AP-1 interactions for use in chemopreventive or antineoplastic therapies.

Material and Methods

Cell lines

Rosalie's transfected lines; MCF-7

Plasmids and transgene cloning

The promoter/reporter constructs ΔColl73:LUC and ERE II TK:LUC and the expression plasmid for the fully wild type human ERα (derived from HEG0) have been previously described. The K206 mutation was introduced into human ERα by

site-directed mutagenesis. The mutation was verified by sequence analysis. _EE K206A was generated using a ??? fragment of ERα.K206A into _EE (originally HE19 needs further explanation?) digested with. ERα.K206A mAF-2 was generated by ????

To construct K14-ERα and K14-ERα.K206A, the FGF coding sequence was removed from ??? by a SmaI digest to generate a K14-driven expression vector. ERα and ERα.K206A were removed from pSG5 expression vectors by an EcoR1 digest and blunt-end ligated into the K14 construct. The identities of the plasmids were confirmed by sequencing. K14-ERα and K14-ERα.K206A fragments were excised enzymatically (enzymes) and purified for oocyte injection (protocol).

Transient transfections

Transient transfections were performed as previously described. Briefly, DNA was transfected into cells by electroporation. Transfection efficiency was monitored by co-transfecting an actin-βgal construct. Relative Light Units represent luciferase light units divided by βgal activity.

Transgenic mice

Transgenic mice were created by microinjecting the K14-ERα.K206A or K14-ERα construct into one-cell FVB/n embryos. At the age of 21 days, pups were genotyped by PCR analysis of tail tip DNA using a β-globin-specific primer pair (5'-AGAAAAGAAGGCATGAACATGG-3' and 5'-GTGAGTTTGGGGACCCTTGAT-TGT-3'). Thermal cycling was carried out at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for 35 cycles in a GeneAmp PCR System 9700 (PE Applied Biosystems, Norwalk, CT). The expected xx bp fragment was visualized on a 2% agarose gel stained with ethidium bromide.

Histopathology and immunohistochemistry

As described previously, mice were sacrificed by perfusion of the ascending aorta with 3.75% paraformaldehyd under Avertin anesthesia. Reproductive tracts were dissected and post-fixed overnight at 4°C. After removal of the posterior vaginal wall, tissues were rinsed in phosphate buffered saline (PBS), dehydrated through graded alcohols and xylene, embedded in paraffin with the cut vaginal surface facing downward, and 5_m sections were stained with hematoxylin/eosin (Sigma).

PCNA IHC was carried out as described previously (. Briefly, 5_m tissue sections were deparaffinized, rehydrated and subjected to antigen retrieval in 10mM citrate buffer, pH 6.0 by microwaving them for two 5-minute high-power pulses. Sections were washed,blocked in 3% bovine albumin (Sigma) in PBS, and subsequently exposed to a 1:200 dilution of mouse anti-PCNA monoclonal antibody (Biogenex). Signal development was performed by using a biotinylated goat anti-mouse IgM secondary antibody (diluted 1:200; Vector), the Vector Elite immunoperoxidase reagent (Vector), and working-strength NovaRed solution (Vector) as a substrate. Sections were counterstained with Gill's #1 hematoxylin (Sigma).

Human ERα IHC was carried out similarly, using a 1:200 dilution (in 0.2% bovine albumin) of anti-human ERα monoclonal antibody D75 (Greene G, Nolan C, Engler J, Jensen E (1980). Monoclonal antibodies to human estrogen receptor. Proc. Natl. Acad. Sci. USA, 77:5115-5119) and biotinylated anti-rat IgG secondary antibody (1:200 in 0.2% bovine albumin; Vector).

Cyclin D1 IHC was performed similar to PCNA IHC, but additionally included blocking of endogenous peroxidase by a 20-minute incubation in 3% hydrogen peroxide in methanol right after dehydration of the tissue. A 1:500 dilution of an anti-Cyclin D polyclonal antibody (Upstate Biotechnology) and a 1:200 dilution of a biotinylated anti-rabbit IgM (Vector) were used. 3,3'-diaminobenzidine (Sigma) served as substrate. Sections were not counterstained.

Western blotting

Mice were sacrificed by cervical dislocation, reproductive tracts dissected and fallopian tubes removed. The remaining tissue was snap-frozen and grinded down in chilled standard lysis buffer (1% NP-40/0.1M NaCl/2mM EDTA/0.1M Tris, pH 8.0 with 1_l/ml aprotinin (Sigma), 10_l/ml polymethyl sulfylfluorid (Sigma), 0.5_l/ml 1M DTT (Sigma), and 10_l/ml NaF (Sigma)). Samples were agitated at 4°C for 60min, then cleaned from cell debris by centrifugation at 14,000rpm/4°C. MCF-7 cells were washed twice with PBS prior to lysis and cleaning of the sample. 20_g whole cell extract/protein sample as well as 5_g Rainbow Standard RPN 800 (Amersham) were separated on a 7.5% SDS/polyacrylamide gel and transferred onto a polyvinylidine difluoride membrane. After blocking with PBS/5% milkpowder/0.1% Tween 20, the filter was hybridized with the polyclonal anti-human ERα antibody HC-20 (Santa Cruz) diluted 1:500 in PBS/0.1% Tween20 at room temperature and horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) diluted 1:2,000 in PBS/0.1% Tween 20. The membrane was thoroughly washed in PBS/0.1% Tween 20 and developed by enhanced chemiluminescence (ECL) (Amersham).

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Figure legends

Fig. 1 A point mutation in ERα, K206A, results in super-activation at an AP-1 site but not at an ERE. ***a, Location*** of the ERα.K206A mutation in the first zinc finger of the DNA binding domain., ***b***, ERα. super-activates at AP-1 but shows no increase of activity at an ERE.). Activity of the mutant and wild type hERs on a reporter gene driven by the human collagenase promoter including its AP-1 site (left) or of an isogenic reporter with an ERE substituted for the AP-1 site (right). c, ERα.K206A super-stimulates AP-1 activity in a variety of cell types. Shown is the fold estrogen induction of the collagenase promoter with AP-1 site in various transfected cells with either the wild type or K206A human ER. d, Mutation of AF-2 abolishes ERα.K206A stimulation at AP-1. Activity of K206A compared with a double mutant K206A: AF-2, on the collagenase promoter driven reporter gene.

Fig. 2 K14-ERα.K206A transgenic mice develop vaginal distortion and hypertrophy with age which cannot be explained by transgene expression levels. ***a***, K14-ERα.K206A transgenic females develop perineal swelling (bottom, 12 months; compare to top, 17-months-old non-transgenic). ***b***, Vaginal enlargement develops in K14-ERα.K206A transgenic (3rd and 4th panels) but not in non-transgenic (1st panel) or wild type ERα transgenic (2nd panel) mice. ***c***, Aged K14-ERα.K206A mice develop redundant and distorted vaginas with hypertrophic squmamous epithelium (top, 20x). This is not due to excessive transgene expression as shown by anti-huERα IHC (antibody: D75): Immunoreactivity in K14-ERα.K206A vaginal epithelium is rarely present in basal cell nuclei, whereas in wild type ERα transgenic vaginal epithelium, huERα immunoreactive basal cells are widespread. Endogenous murine ERα does not cross react with D75 (compare FVB/n to ERα and ERα.K206A) (200x).

Fig. 3 Vaginal epithelial hyperplasia in K14-ERα.K206A transgenic mice is associated with greater numbers of proliferating cells compared to wild type K14-ERα transgenic animals. ***a***, Vaginal and cervical epithelia of K14-ERα.K206A transgenic mice contain more cell layers than do non-transgenic animals (FVB/n). ***b***, In 12-months-old K14-ERα.K206A transgenic vaginal tissue, PCNA

immunoreactivity is more intense than in age-matched wild type ERα and non-transgenic control mice, and the number of positive cell layers can reach ≥ 5 (400x). ***c*** , Cyclin D1 immunoreactivity of 12-months-old K14-ERα.K206A vaginal epithelium is markedly stronger than that of transgenic and non-transgenic control tissues.

Supplement Figure In non-transgenic mice in full estrus, Cyclin D1 immunoreactivity does not reach that seen in K14-ERα.K206A. **a,** Squamous epithelium shows high proliferative activity in mice in estrus as indicated by abundant mitotic cells. ***b,*** In accordance with epithelial proliferation, non-transgenic mice in estrus display a high number of Cyclin D1-positive cells. Staining intensity is comparable to that seen in K14-ERα transgenic mice, but is significantly lower than in K14-ERα.K206A transgenic epithelium (compare Fig. 3*c*). ***c***, Non-transgenic cervical stroma of animals in estrus is devoid of Cyclin D1-positive cells.

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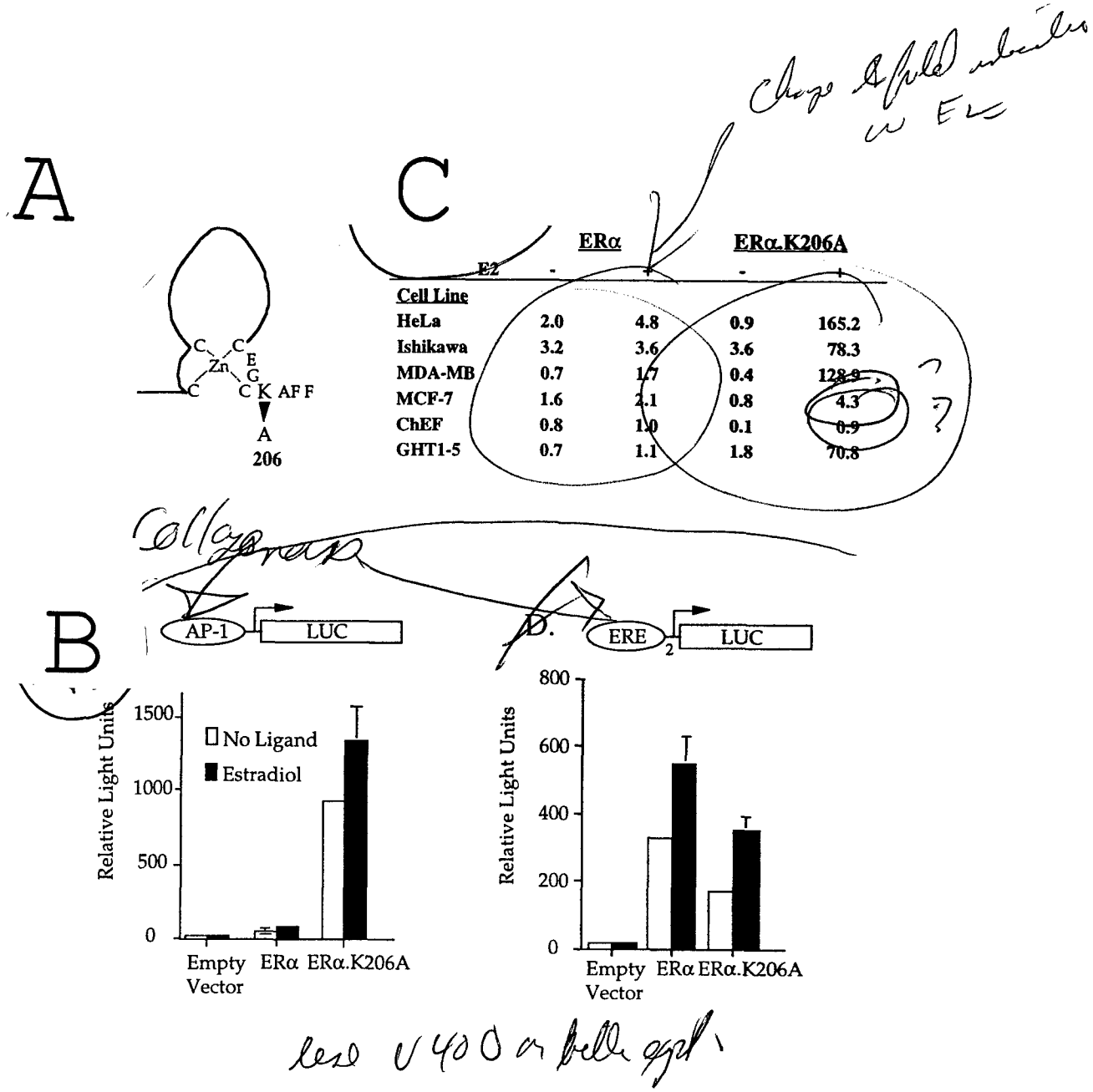
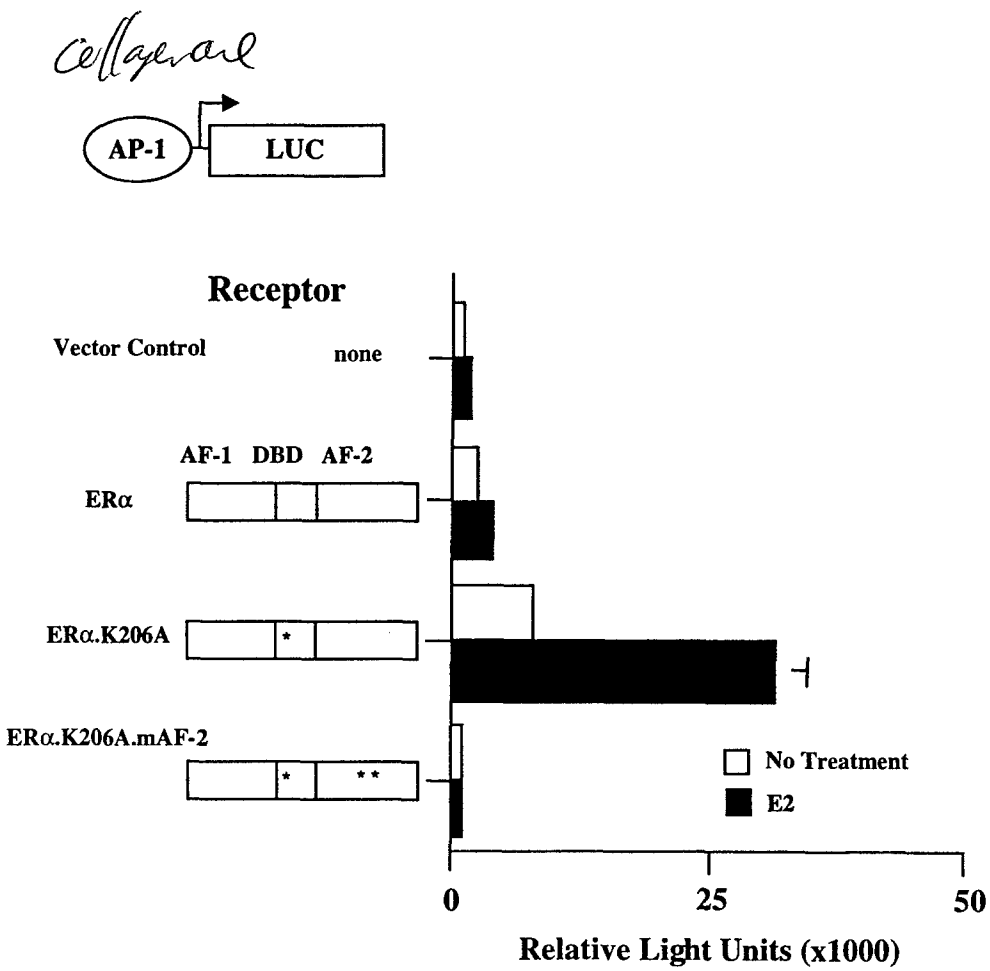
Figure 1 A, B, C



K206A Tgm Ms: Fig. 1

Figure 1D



K206A Tgm Ms: Fig. 2

Figure 2

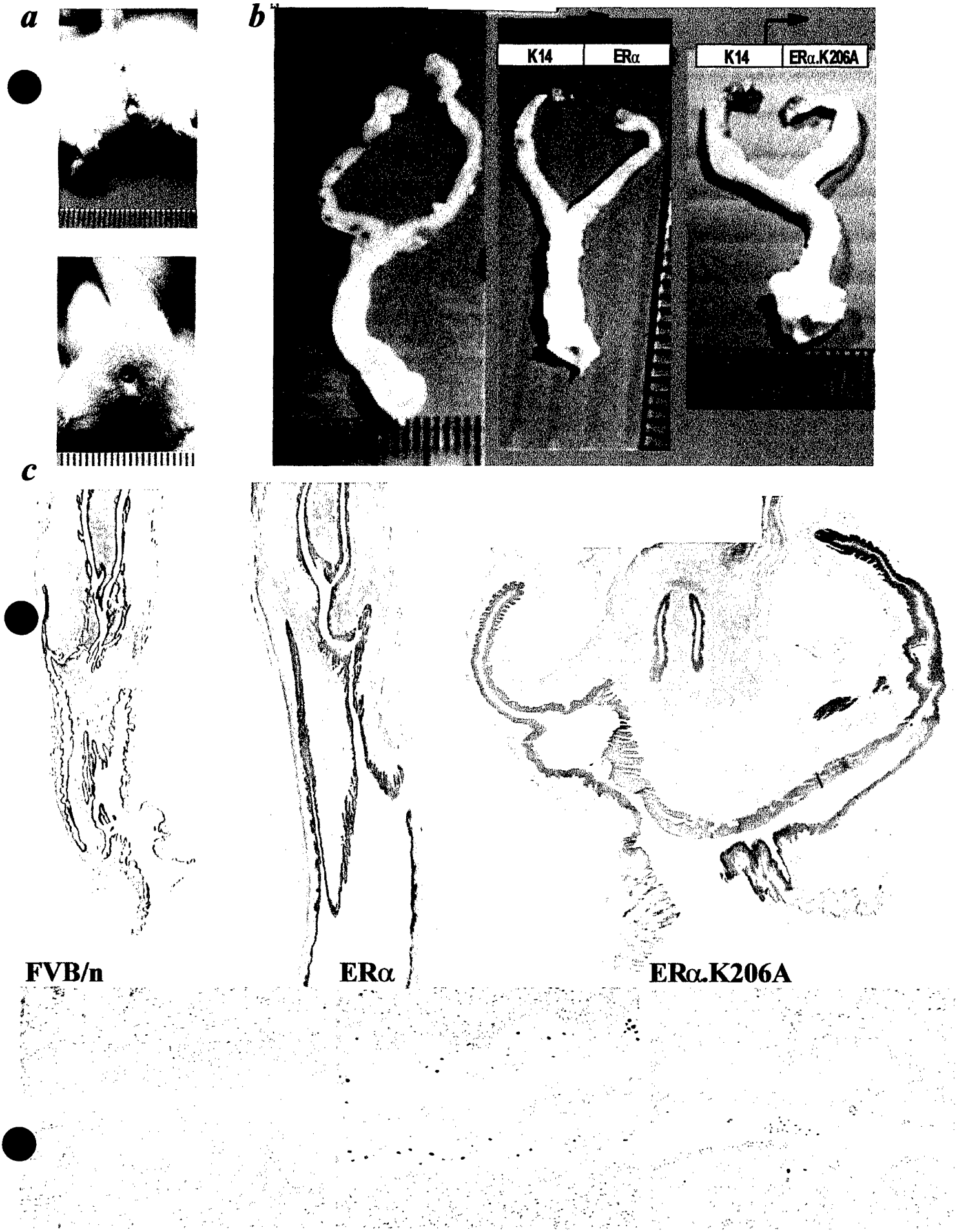
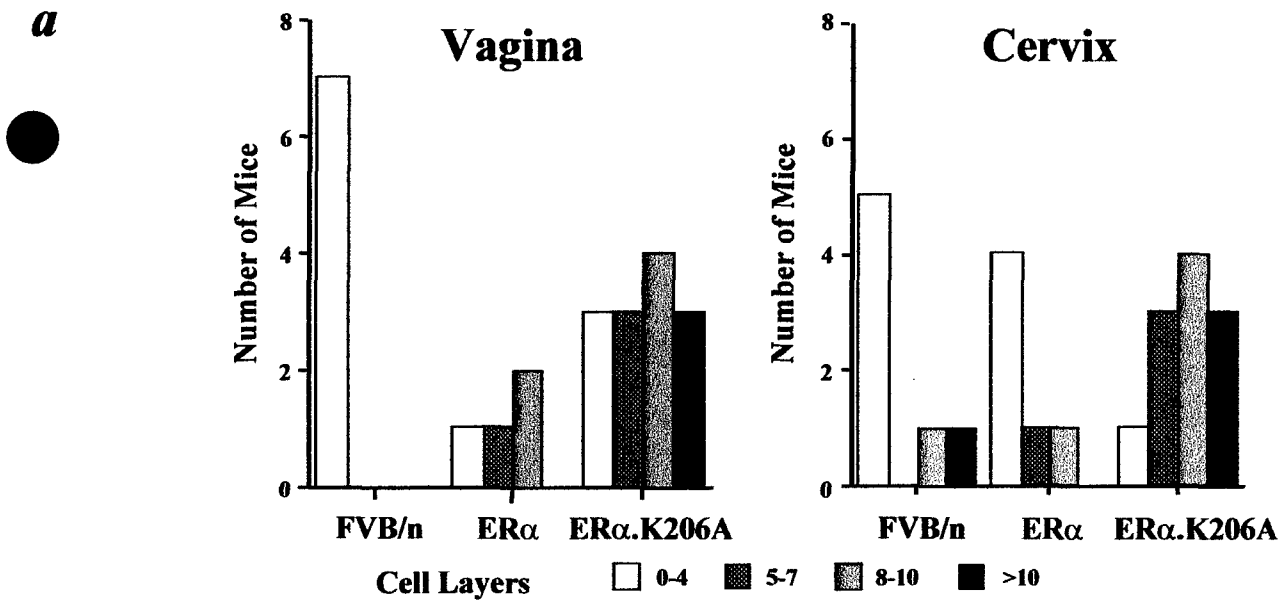
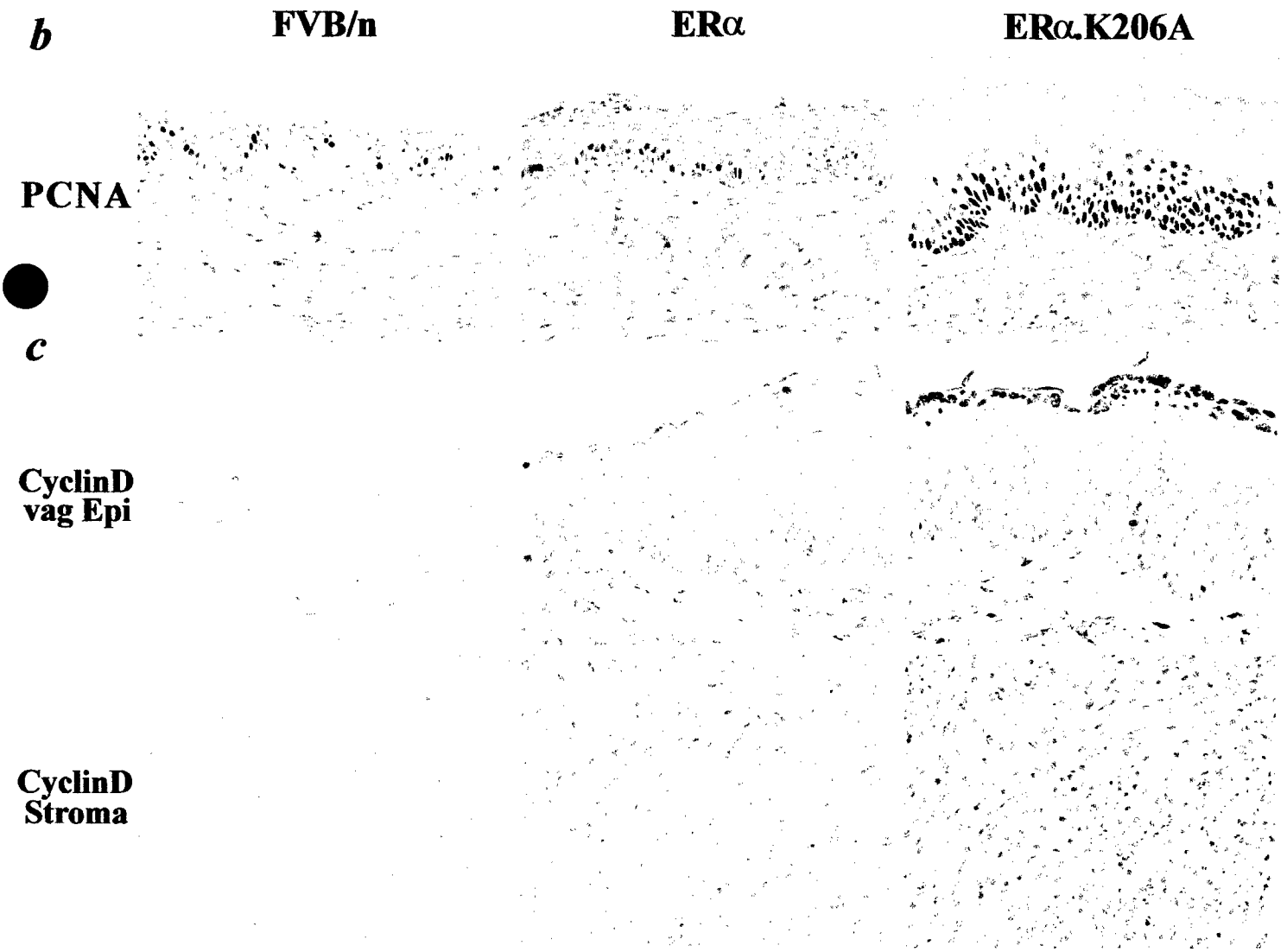


Figure 3


a
Vagina
Cervix
Number of Mice
0
2
4
6
8
FVB/n
ERα
ERα.K206A
Cell Layers
0-4
5-7
8-10
>10


b
FVB/n
ERα
ERα.K206A
PCNA
c
CyclinD
vag Epi
CyclinD
Stroma

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REPLY TO
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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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